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(54) Title: IMPROVEMENTS IN OLIGONUCLEOTIDE PRIMERS AND PROBES

(57) Abstract

This invention relates to oligonucleotides comprising one or more degenerate base analogues of the structure shown in Figure 2 in which R = H or NH_2 and R′ = H or CH_3, for use as primers e.g. in PCR and as hybridisation probes. Also disclosed are methods of performing DNA sequencing reactions or the polymerase chain reaction and hybridisation reactions, involving use of oligonucleotides containing one or more degenerate base analogues of the present invention.

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Title: Improvements in Oligonucleotide Primers and Probes

Field of the Invention

This invention relates to oligonucleotides and their use as primers in DNA sequencing and Polymerase Chain Reactions (PCR) and as hybridisation probes.

Background to the Invention

DNA sequence determination and PCR are both widely-used techniques. Both methods require the use of oligonucleotides which will hybridise to the regions adjacent to and/or including a particular DNA sequence of interest. For PCR in particular, this necessitates the sequence-determination of short sections of DNA flanking and/or including the region of interest. Commonly, this is achieved by determining the amino acid sequence of the peptide encoded by the target sequence to amplified.

However, because of genetic code degeneracy, the nucleotide sequence of the structural gene and the messenger RNA of the organism in which the protein was formed, or a corresponding copy DNA (cDNA), cannot be uniquely defined. Thus the appropriate unique complementary oligonucleotide primers cannot be readily determined. As a hypothetical example, for the following amino acid sequence (1a) the possible messenger RNA (mRNA) (1b) or equivalent cDNA sequences would hybridise best

with one of eight possible oligonucleotides (1c).

... met lys his cys (1a)

... AUG AA^{A}_{G} CA^{U}_{C} UG^{U}_{C} (1b)

TAC TT^{T}_{C} CT^{A}_{G} AC^{A}_{G} (1c)

In practice this problem can be overcome in a number of ways. For instance, multiple primers can be synthesised which correspond to all possible codon assignments. Alternatively, fewer oligonucleotides can be made, based on a codon usage table, allowing for known codon bias of the tissue or organism in question. Additionally, oligonucleotides can be synthesised which incorporate "neutral" bases, such as deoxyinosine (I), which stand in place of A, G, T and C at positions of degeneracy (i.e. those positions where doubt exists over the correct complementary base). However, none of these approaches is ideal. Such methods either require considerable experimental work or have the potential for weakening the hybridisation between primer and DNA sequence under analysis.

Reference 1 discloses a purine base analogue (capable of binding to T and C with comparable affinity) N^{6}- methoxy - 2, 6- diaminopurine (known for convenience as K), the structure of which is shown in Figure 1, and deoxynucleosides and oligonucleotides incorporating K.

The present invention is based on the unexpected discovery that oligonucleotides containing synthetic base analogue K and related bases can be used a primers for DNA sequence determination and PCR and as hybridisation probes.

Summary of the Invention

According to one aspect of the present invention there is provided a method of performing the polymerase chain reaction (PCR), wherein an oligonucleotide used as a primer comprises one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

In another aspect, the invention provides an oligonucleotide for use as a primer in PCR, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

K has the structure shown in Figure 2 with R = NH_2, R' = CH_3. In a related base known as Z, R = H, R' = CH_3. In a modified form of K, R = NH_2, R' = H.

The ability to use such oligonucleotides as primers for PCR is unexpected and could not be predicted. PCR requires use of exceptionally heat-stable DNA polymerase, and the enzyme generally employed in PCR is Taq polymerase from the extreme thermophile Thermus aquaticus. For a base (other than the natural ones) to be capable of use in PCR, primers containing the base must be capable of polymerase chain extension. In addition, in the second phase ("second strand synthesis") the base must be recognised as a base in the extended primer, and there is no reason to expect K and related bases to behave in this way.

Bases of the structure shown in Figure 2 can act as "degenerate" purine analogues in PCR primers, being capable of base-pairing with both C and T with comparable

affinity. Base pairing of K with C and T is illustrated in Figure 3. Use of K and related bases in PCR primers at positions of degeneracy can thus either avoid the need to use multiple primers or reduce significantly the number of different primers required.

Use is conveniently made of PCR primers also incorporating a pyrimidine base analogue, capable of binding to both A and G with comparable affinity. For example the base 3,4-dihydro-8H-pyrimido [4,5-C] [1,2] oxazino-7-one (known for convenience as P), as described in reference 2, and related bases may be used for this purpose, as described in the specification of co-pending British Patent Applications Nos. 9119377.1 and 9123187.8. The neutral degenerate base analogue I may also be used.

A deoxyribonucleoside incorporating K and related bases, as shown in Figure 4, can be used to prepare "monomers", for example as shown in Figure 5a and 5b. Such monomers are capable of being used in an automated DNA synthesiser to prepare oligonucleotides, said oligonucleotides being able to hybridise to a number of different specific sequences of single stranded DNA with comparable efficiency (stringency) and are effective as primers in PCR when hybridised to any one of a number of particular sequences of single stranded or double stranded nucleic acids.

Oligonucleotides comprising one or more K and related bases can also be employed as primers for DNA sequence-determination reactions.

Another aspect of the present invention provides an oligonucleotide for use as a DNA sequencing primer,

comprising one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

A further aspect of the invention provides a process for determining the sequence of a stretch of DNA, comprising the steps of synthesising an oligonucleotide containing one or more bases of the structure shown in Figure 2, wherein R = H or NH_2, R'= H or CH_3, hybridising said oligomer to said DNA and using said oligonucleotide as a primer for chain extension.

It will be apparent to those skilled in the art from the foregoing that such oligonucleotides can also act as effective hybridisation probes for nucleic acid sequences.

Accordingly, in another aspect the invention provides an oligonucleotide for use as a probe in hybridisation reactions e.g. as a probe for the products of PCR, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

In a further aspect, the invention provides a method of performing a hybridisation reaction comprising use of an oligonucleotide comprising one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

Such hybridisation reactions are preferably carried at a temperature at least 3°C below the predicted T_m for the probe. Typically the temperature is in the range of 8-15°C below the predicted T_m.

As described above, oligonucleotides comprising one or more K or related bases most conveniently further comprise one or more degenerate pyrimidine analogues and possibly also one or more neutral degenerate base analogues, whether intended for use as primers or probes.

When used as probes, oligonucleotides according to the invention will generally incorporate a label, in a manner well known to those skilled in the art. Such labels may be, e.g. radiolabels, fluorescent labels or enzyme labels.

Clearly, further modifications of K can be envisaged which are within the scope of the present invention. Such modifications in particular might comprise various substitutions at R and R'.

The invention will be further described by way of illustration and with reference to the accompanying drawings in which:

Figure 1 shows the structure of the base known as K;

Figure 2 shows the general formula of K and related bases;

Figure 3 shows K in its amino and imino tautomeric forms, base-pairing with thymine (T) and cytosine (C) in a Watson-Crick manner;

Figure 4 shows the general formula of a deoxyribonucleoside incorporating the base of Figure 2;

Figures 5a and 5b show formulae of monomer derived fom the

deoxyribonucleoside of Figure 4;

Figure 6 shows reaction schemes used to obtain monomer incorporating K;

Figure 7 illustrates production of monomer incorporating K and another monomer incorporating P;

Figures 8 to 11 show the results of electrophoretic gel separation of PCR - amplified products in which one oligonucleotide primer was the perfect complementary sequence to part of the DNA sequence to be amplified, whilst the other primer contained either the degenerate bases K, P or M (M is a base related to P) at one or more positions or was a positive control (i.e. another perfectly complementary primer); and

Figures 12 and 13 show the results of hybridisation (Southern blot) experiments conducted at 45°C or 32°C using multiple oligonucleotides or oligonucleotides containing degenerate analogues such as K and P or I.

Synthesis of K Monomer

This is illustrated in Figure 6.

One route to N^6-methoxy-2,6-diaminopurine nucleosides is from deoxyguanosine. Sugar-protected O^6-sulphonylated intermediates have been used in nucleophilic displacements at that site (references 3,4). Unfortunately, reaction with methoxyamine was temperamental and, although products of the form 3 (R = HNAcyl) were obtained (reference 5), we preferred the earlier methods involving 6-chloro intermediates (reference 6). The nucleosides

corresponding to 8 and 11 of Figure 6 have also been synthesised by other workers, using a Dimroth rearrangement route (references 7,8).

2-amino-6-chloropurine was coupled in high yield with 2-deoxy-3,5-di-O-p-toluoyl-alpha-D-ribosyl chloride, using the phase-transfer method of Seela and co-workers (references 9,10), which leads to inversion at C-1 with high stereospecificity. The major product was the 9-beta-nucleoside 5a together with a minor product assigned the 7-beta structure 5b. In order to establish the structure of the major product 5a, it was further acylated to the N^2, 3',5'-tri-p-toluoyl derivative 6. Deoxyguanosine (7) was tri-p-toluoylated and then converted, although in poor yield, into the 6-chloro compound 6, identical to that from the glycosylation route.

Conversion of 5a into the N^6-methoxy derivative 8 was best effected by methoxyamine in dry ethanol, following Giner-Sorolla and co-workers (reference 6). We found that, in this series, as with 2, 6-diaminopurine nucleosides, N^2-acyl groups require vigorous conditions for their removal (reference 3). The nucleoside 8 was therefore deacylated and converted into the N^2-dimethylaminomethylene intermediate, in high yield, and thence into the 5'-O-(4,4'- dimethoxytrityl) derivative 9. This product was converted into the 3'-(2-cyanoethyl N, N-di-isopropylphosphoramidite) "monomer" 10a in the normal way (reference 11). Using a similar route from 6-chloropurine, N^6-methoxy-3',5'-di-O-p-toluoyl-deoxyadenosine (11) was obtained and, after deacylation, was converted into the monomer 10b. In the chloropurine coupling reaction, no evidence of a minor regio-isomer was found.

The analogue phosphoramidites 10a and 10b were then used to prepare oligomers by automatic machine synthesis, using the same coupling times used for the normal monomers. They were worked-up in the usual way following treatment with aqueous ammonia, and purified by ion-exchange h.p.l.c. The oligomers synthesised are listed in Table 1, which shows the T_m values of duplexes formed between heptadecamers that contain Z and K and two complementary duplexes that differ only at position 9(T/C). For comparison T_m values of the fully complementary duplexes and others that contain GT and AC mismatches are included.

Inspection of Table 1 shows that the fully complementary duplexes (entries 1,2) are more stable than those containing N^6-methoxyadenine (Z) or N^6-methoxy-2,6-diaminopurine (K). Indeed, a single G.T mismatch (entry 3) reduces the T_m less than does a single Z.T or K.T pair (entries 6, 11). The Z-containing duplexes have uniformly lower T_m values that the K-series. Turning therefore, to the latter series, the original intention of the experiments appears to be borne out, that is, the base pairs K.T and K.C give closely similar contributions to duplex stability. This is seen clearly in entries (11,12), (13,15) and (16,17) in which one, two, and three K-residues are compared. It is evident that the drop in T_m over this series is relatively small and that compared with the triple mismatch (entry 5), those duplexes with three K-residues (entries 16,17) are much more stable.

Experimental Details

General

2-amino-6-chloropurine and 6-chloropurine were purchased from Aldrich Chemical Co. 2-Deoxy-3,5-di-O-p-toluoyl-alpha-D-ribosyl chloride (2-deoxy-3,5-di-O-p-toluoyl-alpha-D-erythro-pentosyl chloride) (in this specification, derivatives of 2-deoxy-D-erythro-pentose are named as derivatives of 2-deoxy-D-ribose) was a generous gift from Dr. R. Hinman and Pfizer Inc., and was also synthesised by the method of Hoffer (reference 12). Flash-column chromatography and t.l.c. were done using Kieselgel 60 H (7736) and 60 F_{254} (Merck), respectively with chloroform-methanol mixtures unless otherwise stated. ^{1}H-N.m.r. spectra (external Me_4Si) were recorded with Bruker WM 250 MHz and AM 400 MHz spectrometers. Mass spectra were recorded with a Kratos M350 instrument, and melting points were measured on an Electrothermal apparatus and are uncorrected.

2-Amino-6-chloro-9-(2-deoxy-3,5-di-O-p-toluoyl-beta-D-ribofuranosyl) purine (5a).

A suspension of finely powdered KOH (3.2g, 58 mmol) and tris [2-(2-methoxyethoxy)ethyl]amine (TDA-1) (0.376g, 1.16 mmol) was stirred in anhydrous acetonitrile (240mL) at room temperature under argon. After 15 min, 2-amino-6-chloropurine (2.0 g, 11.6 mmol) was added and stirring was continued for 10 min. 2-Deoxy-3,5-di-O-p-toluoyl-alpha-D-ribosyl chloride (4.88 g, 12.0 mmol) was added and, after 40 min, the suspension was filtered and taken to dryness. The crude product (6.0 g) was purified by flash-column chromatography, the faster-running major component was collected, and the product (3.22 g, 51%) was crystallised from acetonitrile to give 5a as needles, m.p. 187-188°;

$lambda_{max}$ (95% EtOH) 222, 242, and 310 nm (broad); $epsilon_{nM}$ 4.44, 4.45, and 3.85. ^{1}H-N.m.r. data (Me_2SO): delta 2.37 (s,3H, CH_3), 2.40 (s, 3H, CH_3), 2.69-2.79(m, 1H, H-2'a), 3.19-3.31(m,1 H, H-2'b), 4.51-4.65(m, 3 H, H-4', 5'a, 5'b), 5.73-5.76 (m, 1H, H-3'), 6.40 (t, 1 H, J6.6Hz,H-1'), 7.02(s,2H,NH_2-2), 7.03-7.39(m,4H,Ar), 7.82-7.95(m,4 H, Ar), 8.35(s, 1 H, H-8).

Anal Calc. for $C_{26}H_{24}ClN_5O_5$: C, 59.8; H, 4.6; N, 13.4; m/z (M^+) 521.1427.
Found: C, 59.3; H, 4.8; N, 13.4; M^+ 521. 1485.

2-Amino-5-chloro-7-(2-deoxy-3-5-di-O-p-toluoyl-beta-D-ribofuranosyl) purine (5b).

The slower running, minor component from the chromatography above was collected and the product (0.95 g, 15%) was isolated as a foam with $lambda_{max}$ (95% EtOH) 238 and 322nm (broad) $epsilon_{nm}$ 4.56 and 3.69. ^{1}H-N.m.r. data (Me_2SO): delta 2.36(s, 3 H, CH_3), 2.38 (s, 3 H, CH_3), 2.81-2.91 (m, 1 H, H-2'a), 3.04-3.13(m, 1 H, H-2'b), 4.49-4.64(m, 3 H, H-4',5'a,5'b), 5.69 (t, 1 H, J 3.12 Hz, H-3'), 6.67(t, 1 H, J 6.4 Hz, H-1'), 6.74(s, 2 H, NH_2-2), 7.25-7.37(m, 4 H, Ar), 7.77-7.94(m, 4 H, Ar), 8.72(s, 1 H, H-8).

Anal. Calc. for $C_{25}H_{24}ClN_5O_5$: C, 59.8; H, 4.6; N 13.4; m/z (M^+)521.1427.
Found: C, 59.3; H, 4.5; N, 13.2; M^+ 521.1467.

6-Chloro-9-(2-deoxy-3,5-di-O-p-toluoyl-beta-D-ribofuranosyl)-2-p-toluamidopurine (6).

(a) A solution of 2'-deoxyguanosine (1.0 g, 3.7 mmol) in pyridine (15 mL) was treated dropwise with p-toluoyl chloride (1.71 g, 11.0 mmol). After 8h at 40°C, the solvent was evaporated in vacuo and a solution of the residue in CH_2Cl_2 was washed with aqueous $NaHCO_3$, water, and dried. Removal of the solvent and chromatography gave the tri-p-toluoyl derivative (1.0 g, 43%). ^{1}H-N.m.r. data (Me_2SO): delta 2.36(s, 3 H, CH_3), 2.39(s, 3 H, CH_3), 2.40(s, 3 H, CH_3), 2.74-2.82(m,1 H, H-2'a), 3.18-3.29(m, 1 H, H-2'b), 4.51-4.68(m, 3 H, H-4',5'a,5'b), 5.74 (d, 1 H, J5.6 Hz, H-3'), 6.42-6.49(m, 1 H, H-1'), 7.25-7.40(m, 4 H, Ar), 7.68-7.97(m, 4 H, Ar), 8.28(s, 1 H, H-8), 11.76(b, 1 H, NH), 12.34 (b, 1 H, NH).

To a solution of the above derivative (0.5 g, 0.8 mmol) in dry acetonitrile (10mL) was added tetramethylammonium chloride (0.26 g), N, N-dimethylbenzylamine (0.18mL), and phosphoryl chloride (0.67 mL). The solution was boiled under reflux for 1h, the solvent was evaporated in vacuo, and a solution was washed with aqueous $NaHCO_3$, water, and dried. Removal of the solvent, then chromatography gave 6 as a pale-yellow foam (0.12 g, 23%). ^{1}H-N.m.r. data (Me_2SO): delta 2.35(s, 3 H, CH_3), 2.38 (s,3 H, CH_3), 2.40(s, 3 H, CH_3), 2.76-2.81 (m, 1 H, H-2'a), 3.40-3.46 (m, 1 H, H-2'b), 4.57-4.71 (m, 3 H, H-4', 5'a, 5'b), 5.90 (d, 1 H, J 3.0 Hz, H-3'), 7.24-7.39(m, 4 H, Ar), 7.76-7.96(m, 4 H, Ar), 8.74 (s, 1 H, H-8), 11.23 (s, 1 H, NH).

Anal. Calc. for $C_{34}H_{30}ClN_5O_6$: m/z (M^+)639.1884.
Found: M^+639.1905.

(b) Compound 5a (0.42 g, 0.77 mmol) was treated with p-toluoyl chloride (0.92 g, 0.143 mmol) in anhydrous pyridine (15mL) for 6 h. After the usual work-up, the

product was purified by chromatography to afford 6 as a foam that was identical with the product in (a).

6-Chloro-9-(2-deoxy-3,5-di-O-p-toluoyl-beta-D-ribofuranosyl) purine.

6-Chloro-purine (2.5 g) was treated with 2-deoxy-3,5-di-O-p-toluoyl-alpha-D-ribosyl chloride, as described above for 5a, to give the title compound (4.13 g, 48%), m.p. 119°C (from acetonitrile). ^{1}H-N.m.r. data ($CDCl_3$): delta 2.39 (s, 3 H, CH_3), 2.44 (s, 3 H CH_3), 2.83-2.92(m, 1 H, H-2'a), 3.10-3.22(m, 1 H, H-2'b), 4.61-4.83(m, 3 H, H-4',5'a,5'b), 5.81-5.84 (m, 1 H, H-3'), 6.53-6.59(m, 1 H, H-1'), 7.18-7.29(m, 4 H, Ar), 7.83-7.98(m, 4 H, Ar), 8.28(s, 1 H, H-8), 8.66(s, 1 H, H-2).

Anal. Calc. for $C_{26}H_{23}ClN_4O_5$: C, 61.6; H, 45; N, 11.1; m/z (M^+)506.1356.
Found C, 61.3; H, 4.5; N, 10.9; M^+506.1311.

2-Amino-9-(2-deoxy-3,5-di-O-p-toluoyl-beta-D-ribofuranosyl)-6-methoxyaminopurine (8).

To a solution of 5a (0.2 g, 0.368 mmol) in dry EtOH (2 mL) was added methoxymine (0.5 mL), and the sealed vessel was heated at 90° for 4h. The solvent was evaporated and a solution of the product in $CHCl_3$ was chromotagraphed to give 8 (100mg, 49%) as a foam. ^{1}H-N.m.r. data (Me_2SO): delta 2.38(s, 3 H, CH_3) 2.40(s, 3 H, CH_3 2.62-2.70(m, 1 H, H-2'a), 3.00-3.12(m, 1 H, H-2'b), 3.73(s, 3 H, $NOCH_3$), 4.46-4.64(m, 3 H, H-4',5'a, 5'b), 5.66-5.69(m, 1 H,H-3'), 6.18-6.24(m, 1 H, H-1'), 6.58 (b, 2 H, NH_2), 7.31-7.38(m,4 H, Ar), 7.72(s, 1 H, H-8), 7.86-7.98(m, 4 H, Ar), 9.84(s, 1 H, NH).

Anal. Calc. for $C_{27}H_{28}N_6O_6$:m/z(M^+)532.2070.
Found: M^+532.2025.

2'-Deoxy-N^6-methoxy-3',5'-di-O-p-toluoyladenosine (11)

6-Chloro-9-(2-deoxy-3,5-di-O-p-toluoyl-beta-D-ribonfuranosyl) purine was treated with methoxyamine in dry EtOH, as described above, to obtain 11, as a colourless crystalline powder, m.p. 213°C. ^{1}H-N.m.r. data (Me,SO): imino tautomer, delta 2.38(s, 3 H, CH_3), 2.40(s,3 H, CH_3), 2.68-2.75(m, 1 H, H-2'a), 3.16-3.21(m,1 H H-2'b), 3.76(s, 3 H, $NOCH_3$, 4.49-4.62(m, 3 H, H-4',5'a, 5'b), 5.75(b, 1 H, H-3'), 6.38(t,1 H J6.6Hz, H-1'), 7.30-7.38(m,4H,Ar), 7.49(s, 1 H, H-2), 7.84-7.95(m,4 H, Ar), 8.07(s, 1 H, H-8), 11.25(b, 1 H, NH); amino tautomer, delta 5.61(b, H-3'), 6.54(b, H-1'), 8.27(s, H-8), 8.42(s H-2), 11.00(b,NH).

Anal. Calc. for $C_{27}H_{27}N_5O_6$:m/z(M^+)517.1961.
Found: M^+517.1972.

9-[2-Deoxy-5-O-(4,4'-dimethoxytrityl)-beta-D-ribofuranosyl]-2-dimethylaminomethyleneamino-6-methoxyaminopurine 3'-(2-cyanoethyl N,N-di-isopropylphosphoramidite (10a)

Compound 8 was heated at 55°C overnight with saturated NH_3/MeOH to give the free nucleoside quantitatively, a solution of which (0.4 g, 1.35 mmol) in anhydrous N,N-dimethylformamide (2.5mL) and N,N-dimethylformamide dimethylacetal (2.5 mL) was stirred at 50°C for 2 h. Removal of the solvent and further coevaporation of toluene and acetone from the residue in vacuo gave the N^2-

dimethylamino-methylene derivative (one spot in t.l.c.). The crude product was treated with 4,4'-dimethoxytrityl chloride (0.54 g, 1.62 mmol) in dry pyridine at room temperature for 1.5h. Removal of the solvent in vacuo then chromatography of the dark-blue foam with CH_2Cl_2-Me_2CO(4:1) afforded the dimethoxytrityl derivative 9 (131 mg) as a pale-yellow foam. ^1H-N.m.r. data (Me_2SO): delta 3.01(s, 3 H, NCH_3), 3.10(s, 3 H, NCH_3), 3.72(s, 9 H, 3 OCH_3), 6.77-7.36(m, 13 H, Ar), 7.77(s, 1 H, H-8), 8.48(s, 1 H, N = CHN), 8.88(s, 1 H, NH).

A solution of 9 (120 mg, 0.19 mmol) in anhydrous tetrahydrofuran (5 mL) and Hunig's base (0.132mL, 7.6mmol) was treated, with the exclusion of moisture, with 2-cyanoethyl N, N-diisopropylphosphoramidochloridite (0.66 mL, 0.285 mmol). Reaction was complete in 1 h. The solution was diluted with ethyl acetate, washed with saturated aqueous NaCl, and dried (Na_2SO_4). Chromatography of the product with ethyl acetate-CH_2Cl_2-Et_3N (45:45:10) afforded 10a (115 mg, 73%) ^{31}P-N.m.r. data ($CDCl_3$): delta 148.93 and 149.15.

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N^6-methoxyadenosine 3'-(2-cyanoethyl N,N-di-isopropylphosphoramidite) (10b).

Compound 11 was deacylated to the free nucleoside, which was converted into the 5'-O-(4,4'-dimethoxytrityl) derivative. This product was chromatographed and the pure compound (51% yield) was converted, as described above, into 10b. ^{31}P-N.m.r. data ($CDCl_3$): delta 149.08 and 149.22.

Synthesis of Oligonucleotides.

The phosphoramidite monomers 10a and 10b were incorporated into oligonucleotides, using the normal programme on applied Biosystems 380B and Pharmacia gene assembler Instruments. Deprotection was complete after treatment with conc. aqueous NH_3 at 55°C overnight. Purification was carried out by h.p.l.c. on a Waters system, using a Whatman SAX Partisphere column and potassium phosphate (pH 6.6) gradient in aqueous 60% formamide. The oligomers synthesised are listed in Table 1.

Melting Transitions of Oligonucleotide Duplexes

Melting transitions were measured at 260 m in 6xSSC buffer at an oligomer strand concentration of about 3um. Absorbance vs. temperature for each duplex was obtained using a Unicam SP500 spectrometer (Pye Unicam, Cambridge, U.K.) fitted with a Gilford 222 photometer and 2527 thermoprogrammer (Gilford Instruments, Oberli, OH). The temperature was increased by 1°/min and melting temperatures (T_m) were determined as the midpoints of the sigmoidal melting curves with an error of + or - 1°C.

Synthesis of functionalised controlled pore glass (CPG) support carrying the 3'-O-succinate of (5-dimethoxytrityl-2-deoxyribofuranosyl)-3,4-dihydro-8H-primido [4,5-C][1,2] oxaxin-7-one (2).

P was synthesised as described in reference 2.

As illustrated in Figure 7, the 5'-dimethoxytrityl derivative (1 in Figure 7) of the nucleoside (P) (60mg) was treated in dry pyridine with succinic anhydride (50mg) and 4-dimethylaminopyridine (10mg) for four days. The 3'-

O-succinate was purified by chromatography and converted to its 4-nitrophenyl ester by reaction with 4-nitrophenol and dicyclohexylcarbodi-imide. The nitrophenyl ester (45mg) with triethylamine (0.2ml) in dimethylformamide (DMF) was shaken with vacuum-dried aminoalkyl CPG (Pierce Inc.) for 24 hours. The CPG was washed with DMF, ether and dried then treated with acetic anhydride in pyridine for 10 minutes, then washed and dried as before. the nucleoside loading of the functionalised CPG(2) was 57.2 umol./g.

Oligonucleotide Synthesis

Oligonucleotides were synthesised using an Applied Biosystems Instrument with the normal synthesis cycle. In addition to the normal protected nucleoside-3' (N,N-diisopropyl cyanoethyl) phosphoramidite monomers, the corresponding phosphoramidites of the nucleosides carrying the pyrimidine bases P (3 in Figure 7) and M (M is a modified version of P - see reference 2), and the purine K (4 in Figure 7) were used in the synthesis of oligodeoxyribonucleotides.

In other applications the CPG functionalised with the dimethoxytrityl derivative of the nucleoside P was used to provide oligonucleotides having the P nucleoside at the 3'-end of the oligomer.

Oligomers were purified by hplc using an ion-exchange column in the usual way.

Representative oligonucleotide sequences are given in the Figures 8 to 11. These have one or more of the bases P, M and K alone (Figures 8 to 11) and P and K together

(Figures 10 and 11).

The use of oligodeoxyribonucleotides containing degenerate bases P, M and K as primers in the polymerase chain reaction (PCR)

The following examples illustrate the usefulness of the present invention. In general, single stranded bacteriophage M13 DNA which contained an insert corresponding to the Tyr Ts gene of B. stearothermophilus (reference 13) was used. The standard PCR protocol was used and the thermal cycle optimised as described (reference 14). Thus, for PCR, a Techne programmable Dri-Block PHC-1 apparatus was used. Each 100ul reaction contained 1um DNA, 200 pmol. of each primer, 4U of Taq DNA polymerase in the recommended buffer (reference 15). Typical thermal cycles were: denaturing temp. 92°C for 1 min; annealing temp. 36-44°C for 1 min.; chain extension temp. 62-70°C for 1 min. and the number of cycles was 30. The regions of the gene to be amplified are shown in the Figures. After amplification the products were electrophoretically separated on standard agarose mini-gels. Representative results are shown in Figures 8 to 11.

In Figures 8 to 11 the sequences of the DNA at which the oligomers prime are shown, together with the primer sequences and the base pairs (e.g. P/A) that the degenerate bases form on hybridisation to the template DNA. Thus P is hybridised to A and to G (as is M) and K is hybridised to T and to C. Fully complementary (perfect) primers are used as positive controls; negative controls have one primer missing. The amplified DNA in each case is shown to have the correct chain length by

reference to restriction nuclease HaeIII digest of ØX174 DNA.

Figure 8 shows that a primer with a 3'-terminal degenerate pyrimidine analogue P base, (lane 3) can be chain extended by Taq polymerase and that P (lane 6) is more suitable than the M alternative pyrimidine analogue (lane 5) for inclusion in PCR primers.

Figure 9 shows that a primer containing three P bases (lane 4) is almost as effective at priming chain extension by PCR as the perfectly complementary primer (lane 1), whilst a primer containing three mis-matched bases (lane 3) is ineffective.

Figure 10 shows that oligomers containing up to three degenerate purine analogue bases ('K') are also effective as PCR primers (lane 3) and that oligomers containing both P and K bases (lanes 4, 5 and 6) are effective as PCR primers.

Figure 11 shows that oligomers containing both P and K bases can be used simultaneously as forward and reverse primers (lanes 4 and 6) to achieve PCR amplification.

It is known from the prior art (reference 15) that oligonucleotides containing P or I can be used as probes in dot blots. Further experiments described herein were conducted to investigate the usefulness of oligonucleotides containing degenerate analogues as hybridisation probes in Southern blots.

The amino acid sequence of a protein, part of NADH/ubiquinone oxidoreductase, was determined, as

described by Walker et al., (reference 16). The protein is termed ASHI, from the first four amino acids of the N terminal. The N terminal amino acid sequence of ASHI is shown below, using the conventional single letter notation:

A S H I T K D M L P G P Y P K T P E E R

F P R

The nucleic acid sequence encoding amino acids H(3) to R(20) was amplified by PCR using forward (F) and reverse (R) primers as described in reference 16. The products of the PCR were separated on an agarose gel and blotted onto an inert matrix (Hybond-N, Amersham International, UK) using conventional techniques. These blots were then subjected to hybridisation experiments using oligonucleotide probes complementary to the nucleic acid sequence acid encoding the 6 middle amino acids.

A number of oligonucleotide probes were used. These included probe P, which comprised multiple (1024) oligonucleotides covering all possible codon assignments. The sequence is shown below where R = A and G, Y = C and T, N = A, G, C and T.

P = YTN CCN GGN CCN TAY CC, T_m = 50°C.

The T_m was calculated for each probe by adding 4°C for each G or C base, 2°C for each T or A and nothing for each P, K or I base analogue.

Another probe, P(P + K), comprised a lesser number of mixed oligonucleotides (96) comprising P and K. The sequence

is shown below:

$P(P + K)$ = PTN CC^{Y}_{K} GG^{R}_{K} CCN TAP CC T_m = 42°C.

Another probe P(P + I), with lower complexity (48), comprised oligonucleotides containing P and the degenerate 'neutral' analogue I. The sequence is shown below:

$P(P+I)$ = PTN CC^{R}_{I} GG^{R}_{I} CC^{Y}_{I} TAP CC, T_m = 40°C.

Figure 12a shows the results of a Southern blot of PCR-amplified fragments encoding the N terminal amino acid of the ASHI subunit. The blot was probed with probe P at a temperature of 45°C. Faint, hybridising bands of the expected size were observed.

Figure 12b shows the results obtained when the same blot was probed, at the same temperature, with probe P(P + K). The signal returned from the hybridising bands was fainter than that obtained when using probe P. This is presumably because 45°C is above the expected T_m of 42°C.

Figure 13a shows the results yielded by using probe P at a lower temperature (32°C). As expected, the hybridising bands show up more clearly.

Similarly, in Figure 13b, which shows a blot probed with probe P(P + K) at 32°C, the signal from the hybridising bands is stronger. Surprisingly however, the strength of signal is dramatically increased whilst the background is only slightly enhanced.

This dramatic enhancement of the signal: noise ratio is even clearer when using probe P(P + I), as shown by Figure

13c. The hybridising bands are extremely dark whilst there is virtually no background and fewer spurious bands are observed.

The results thus demonstrate that:

a) Solid supports for oligonucleotides functionalised with degenerate bases can be prepared.

b) Oligomers with a degenerate base at the 3'-terminus can act as primers for DNA chain extension and be incorporated.

c) Degenerate bases in oligomers so incorporated are recognised by the polymerase in the second strand synthesis and lead to DNA amplification.

d) Oligomers with several degenerate bases including both purines and pyrimidines are effective primers.

e) Oligomers referred to under (c) and (d) are effective primers when corresponding oligomers forming Watson-Crick mismatches do not lead to amplification.

f) Oligomers containing degenerate base analogue K may be used as hybridisation probes and are surprisingly more effective than conventional "mixed" probes, especially when comprising at least one further base analogue.

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TABLE 1

1.	ACTTGGCCGCCATTTTG TGAACCGGCGGTAAAAC	75°
2.	ACTTGGCCACCATTTTG ————T————	72°
3.	ACTTGGCCGCCATTTTG ————T————	70°
4.	ACTTGGCCACCATTTTG ————C————	64°
5.	ACTTGGCCACCATTTTG ——T——C—C———	43°
6.	ACTTGGCCZCCATTTTG TGAACCGGTGGTAAAAC	65°
7.	ACTTGGCCZCCATTTTG ————C————	64°
8.	ACTTGGCCZCCZTTTTG ————T—T———	61°
9.	ACTTGGCCZCCZTTTTG ---————C—T———	57°
10.	ACTTGZCCZCCATTTTG --------C---C————	52°
11.	ACTTGGCCKCCATTTTG TGAACCGGTGGTAAAAC	67°
12.	ACTTGGCCKCCATTTTG ————C————	66°
13.	ACTTGGCCKCCKTTTTG ————T—T———	64°
14.	ACTTGGCCKCCKTTTTG ————C—T———	62°
15.	ACTTGKCCKCCATTTTG ———C—C————	59°
16.	ACTTGKCCKCCKTTTTG ———C—T—T------	58°
17.	ACTTGKCCKCCKTTTTG ——— C—-C—-T--------	57°

Claims:

1. A method of performing DNA sequencing reactions or the polymerase chain reaction (PCR), wherein an oligonucleotide used as a primer comprises one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 and R' = H or CH_3.

2. A method of performing a nucleic acid hybridisation reaction comprising use of an oligonucleotide comprising one or more bases of the structure shown in Figure 2, wherein R = H or NH_2 or R' = H or CH_3.

3. A method according to claim 1 or 2, wherein said oligonucleotide comprises at least one other degenerate base analogue.

4. A method according to claim 3, wherein said further degenerate base analogue comprises P (as herein defined) or deoxyinosine.

5. An oligonucleotide for use as a primer in DNA sequencing reactions or PCR or for use as a probe in nucleic acid hybridisation reactions, wherein said oligonucleotide comprises one or more bases of the structure shown in Figure 2, where R = H or NH_2 and R' = H or CH_3.

6. An oligonucleotide according to claim 5, further comprising at least one other degenerate base analogue.

7. An oligonucleotide according to claim 6, wherein said further degenerate base analogue comprises P (as herein defined) or deoxyinosine.

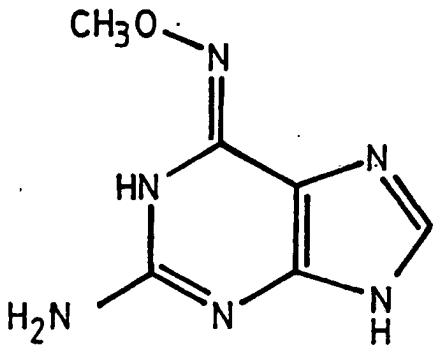


Fig. 1

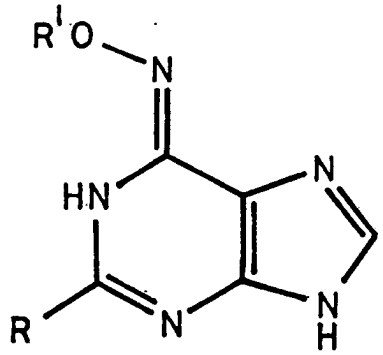


Fig. 2

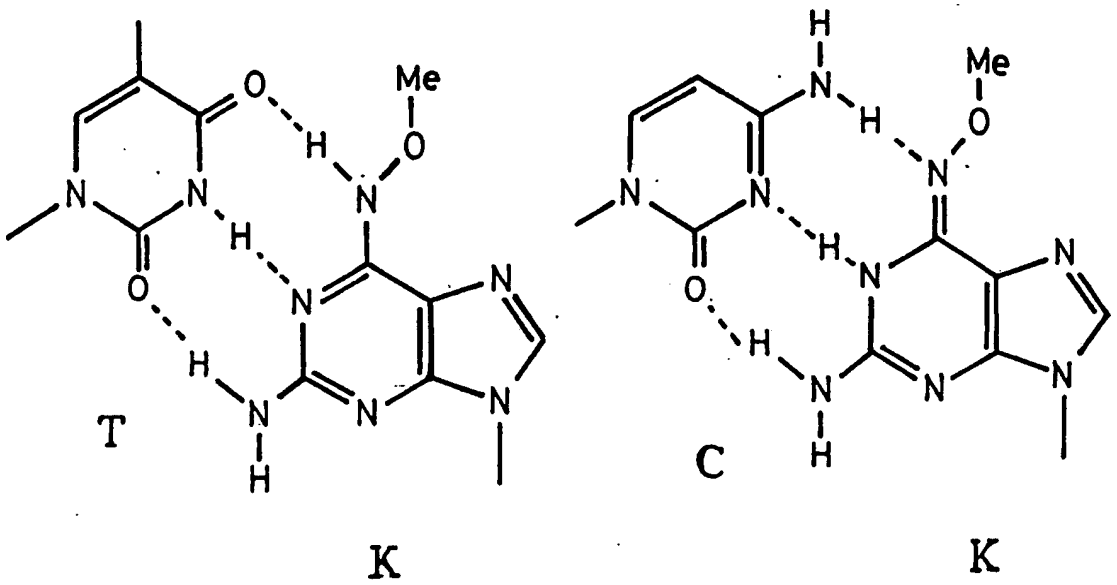


Fig. 3

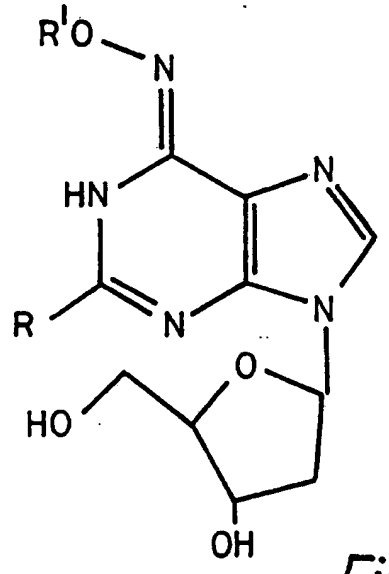


Fig. 4

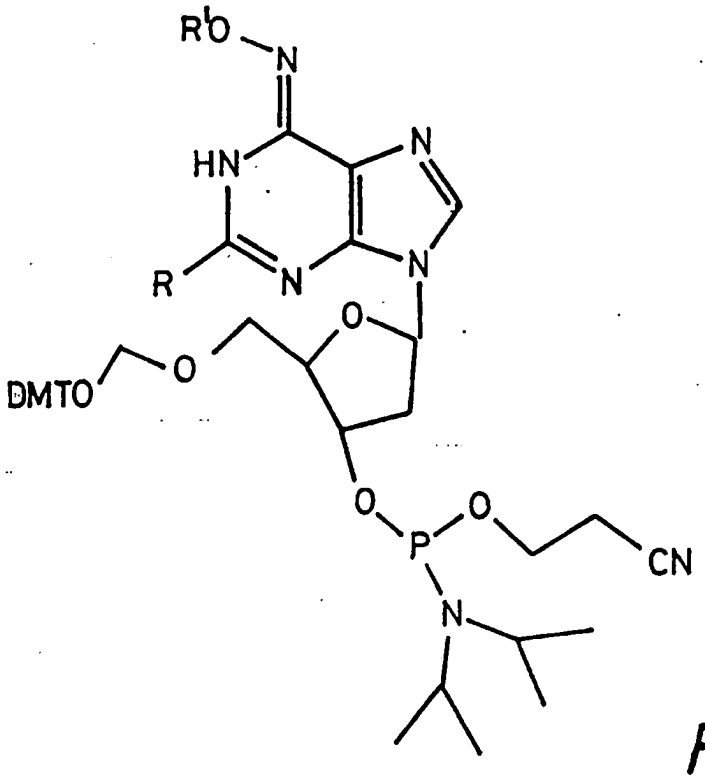


Fig. 5a

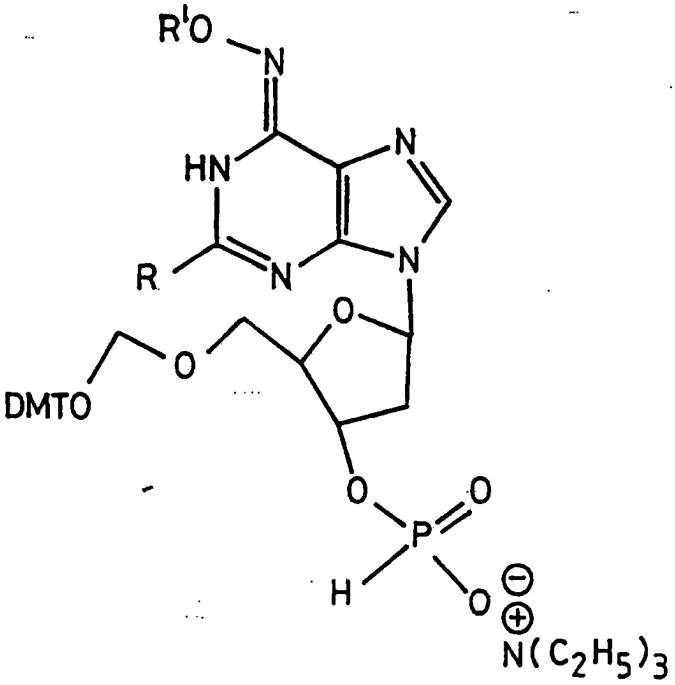


Fig. 5b

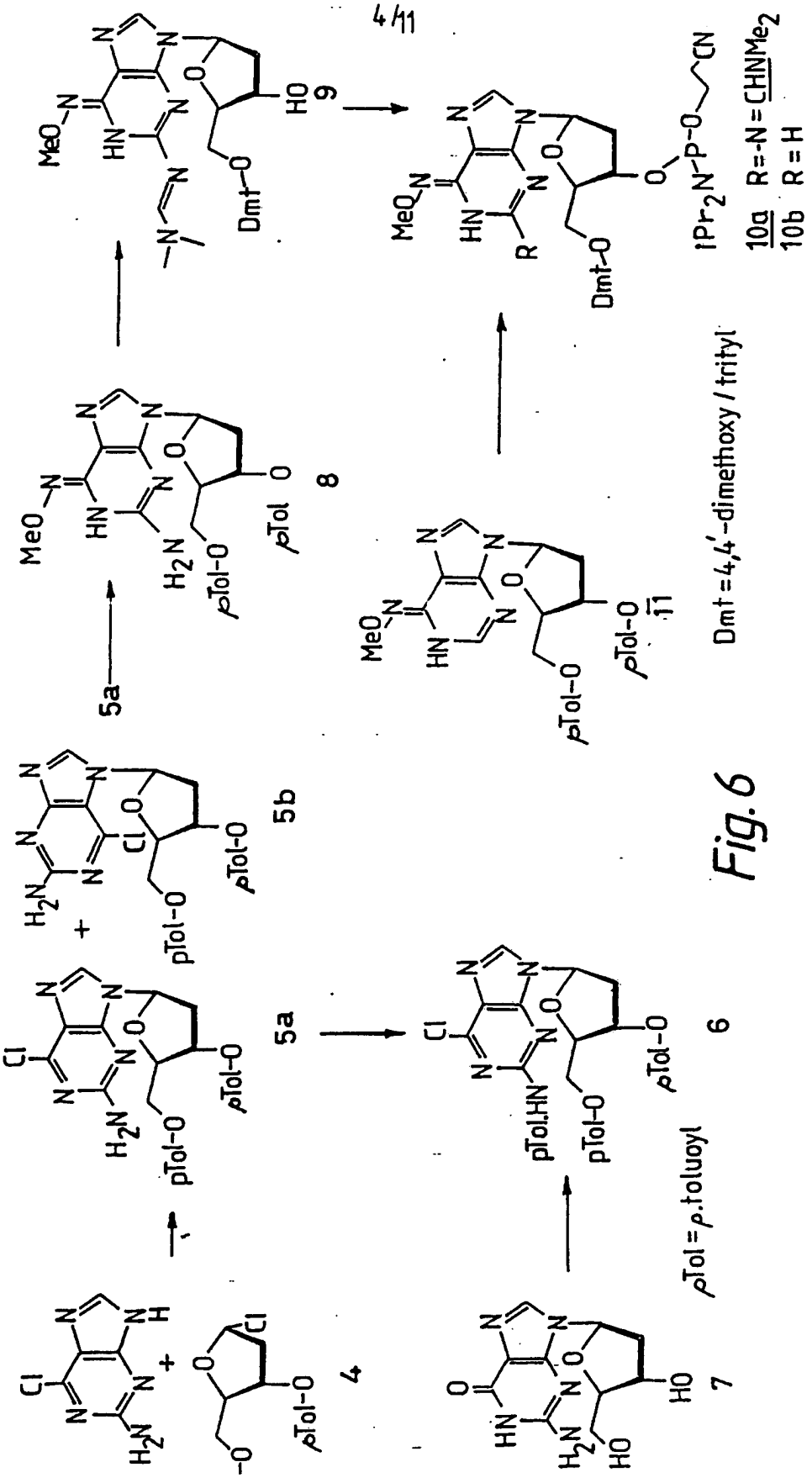
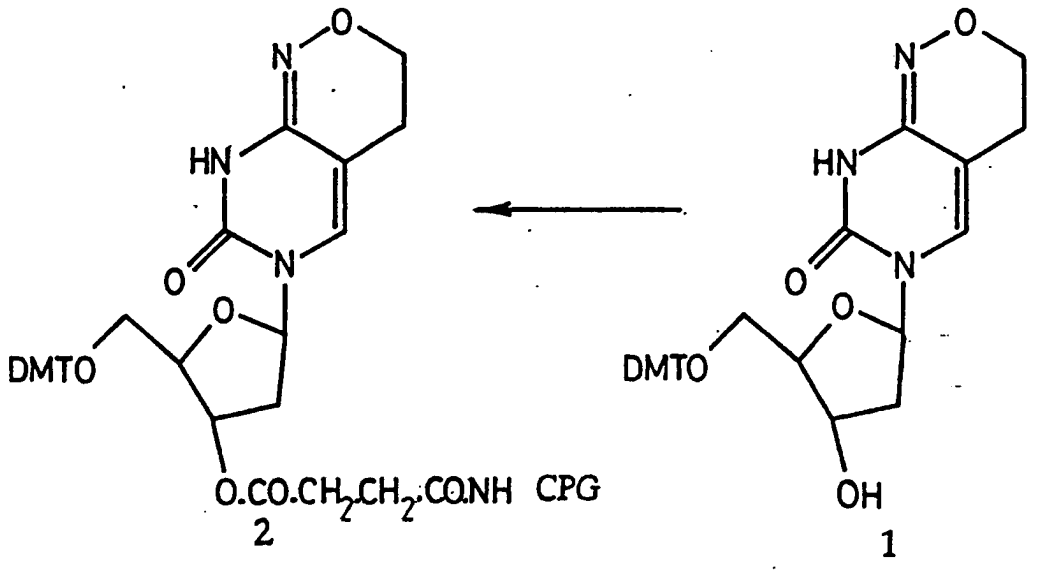

MeO
HN
N
O
HO
9
Dmt-O
Dmt = 4,4'-dimethoxy / trityl
pTol = p.toluoyl
10a R=-N=CHNMe2
10b R = H
iPr2N-P-O-CN
8
pTol-O
5a
5b
11
6
7
4
Cl
H2N
pTol.HN

Fig. 6


DMTO
O.CO.CH2.CH2.CO.NH CPG
2
DMTO
OH
1

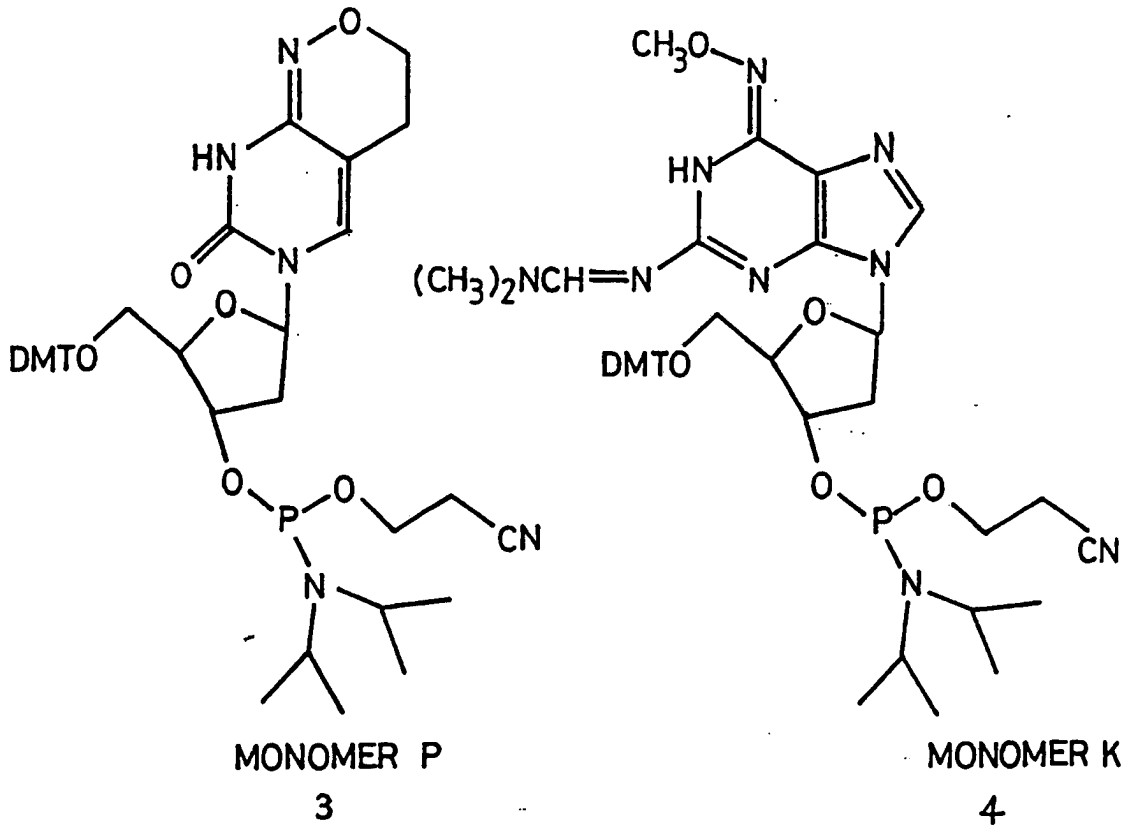

DMTO
CN
MONOMER P
3
CH3O
(CH3)2NCH=N
DMTO
CN
MONOMER K
4

Fig. 7

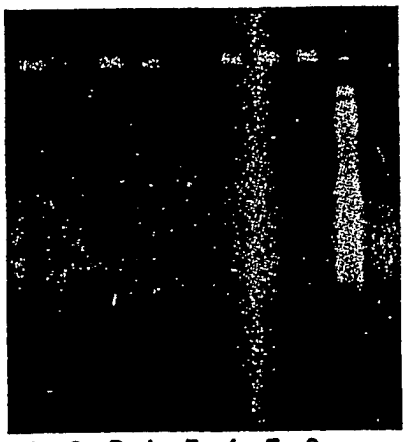
Forward primer 1 5'-GACGGATGAAGACGGGT-3'

5'----------CAAACGACGGATGAAGACGGGTTGCGCAAACTGTTAAAT----

Reverse Primers

2	3'-TGAAPCGGTGGPAAAAC-5'	P/G, P/A
3	3'-TGAAPCGGPGGPAAAAC-5'	P/G, P/A, P/A
4	3'-TGAAMCGGTGGMAAAAC-5'	M/G, M/A
5	3'-TGAACCGGTGGTAAAAM-5'	M/G
6	3'-MGAACCGGTGGTAAAAC-5'	M/A
7	3'-PGAACCGGTGGTAAAAC-5'	P/A
8	3'-TGAACCGGMGGTAAAAM-5'	M/A, M/G
9	3'-TGAACCGGTGGTAAAAC-5'	PERFECT PRIMER

---------GGCCACTTGGCCACCATTTTGACGATGCGCCGCTTCC---------3'



LANE 1 2 3 4 5 6 7 8

PRIMERS 1 1 1 1 1 1 1 1
5 6 7 8 4 2 3 9

Fig. 8

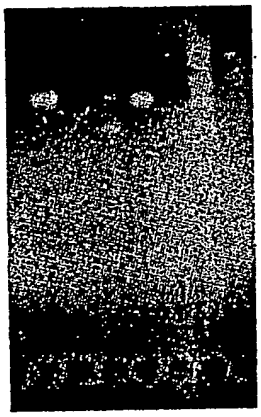
Forward Primer 1 5'-GACGGATGAAGACGGGT-3' Perfect Primer

5'---CAAACGACGGATGAAGACGGGTTGCGCAAACT-------

Reverse Primers

2 3'-TGAATCGGCGGCAAAAC-5' G/T, A/C, A/C
3 3'-TGAAPCGGPGGPAAAAC-5' G/P, A/P, A/P
4 3'-TGAAMCGGMGGMAAAAC-5' G/M, A/M, A/M
5 3'-TGAACCGGTGGTAAAAC-5' Perfect Primer

---------GGCCACTTGGCCACCATTTTGACGAT---------3'



LANE 1 2 3 4 5
PRIMERS {1 1 1 1 1
{5 4 2 3 –

Fig. 9

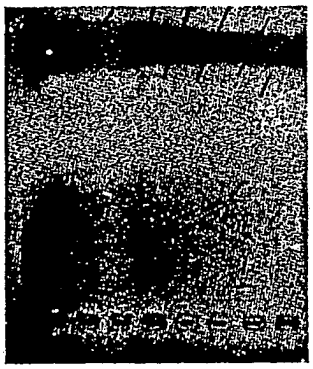
5'----------ACTTGGCCACCATTTTG---------156 BASES

FORWARD PRIMERS

1	5'-ACTTGGCCACCATTTTG-3'	PERFECT PRIMER
2	5'-ACTTGGCCKCCKTTTTG-3'	K/T, K/T
3	5'-ACTTGKCCKCCKTTTTG-3'	K/C, K/T, K/T
4	5'-ACPTGKCCACCKTTPTG-3'	P/A, K/C, K/T, P/ A
5	5'-ACTTGKCCKCPATTPTG-3'	K/C, K/T, P/G, P/A
6	5'-ACPTGKCCACPATTPTG-3'	P/A, K/T, P/G, P/A

----------CGGCCGCTTTTTAGATT----------3'

REVERSE PRIMER 7 3'-GCCGGCGAAAAATCTAA-5' PERFECT PRIMER



LANE	1	2	3	4	5	6	7
PRIMERS	1	2	3	4	5	6	1
	7	7	7	7	7	7	-

Fig. 10

5'---ACTTGGCCACCATTTTG----------

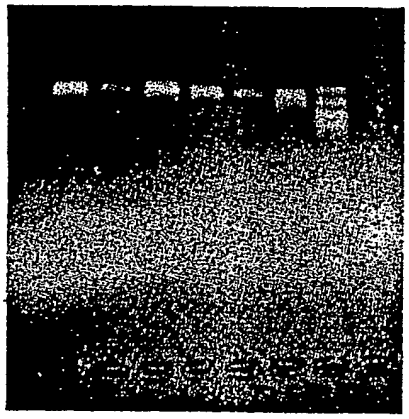
FORWARD PRIMERS

1	5'-ACTTGGCCACCATTTTG-3'	PERFECT PRIMER
2	5'-ACPTGKCCACCKTTPTG-3'	P/A, K/C, K/T, P/A
3	5'-ACTTGKCCKCPATTPTG-3'	K/C, K/T, P/G, P/A

----------GAAGCGGACGGCAATCC----------3'

REVERSE PRIMERS

4	3'-CTTCGCCTGCCGTTAGG-5'	PERFECT PRIMER
5	3'-CTPCGPCTKCCGTTKGG-5'	P/A, P/G, K/C, K/T



LANE	1	2	3	4	5	6
PRIMERS	1	1	2	2	3	3
	4	5	4	5	4	5

Fig. 11

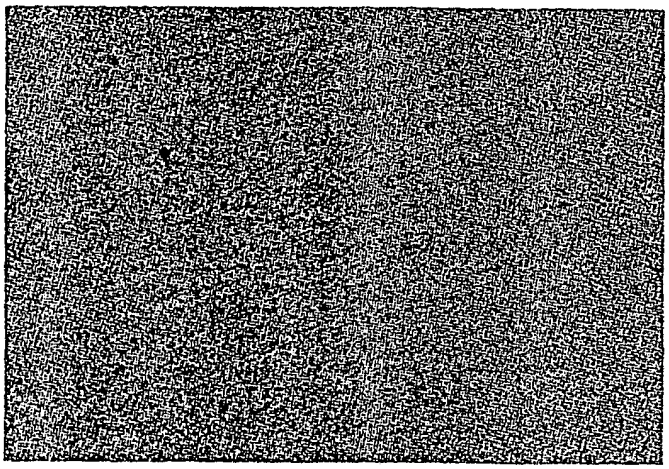


Fig. 12a

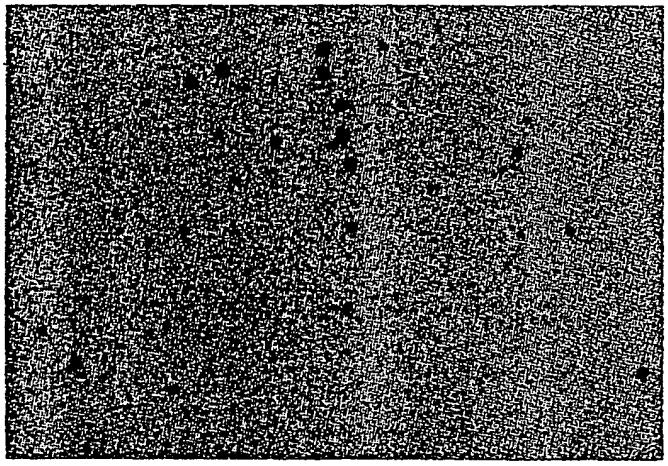


Fig. 12b

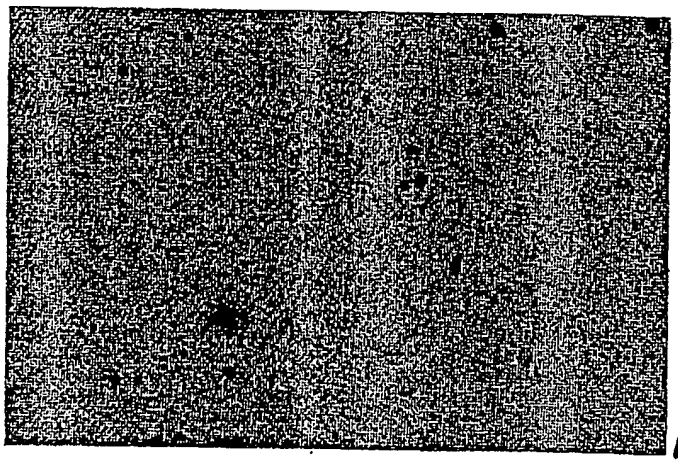


Fig.13a

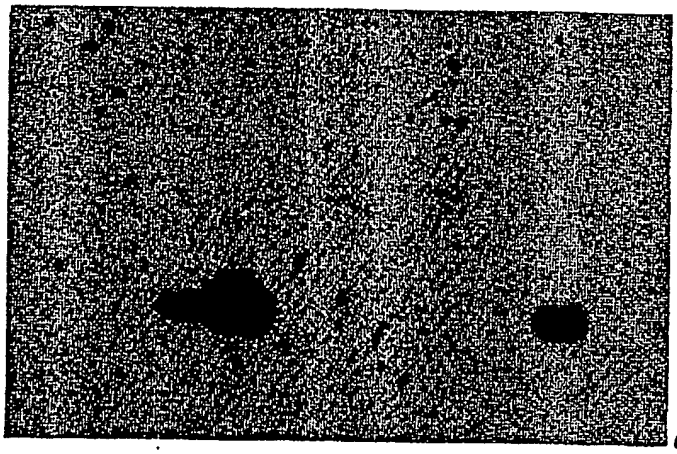


Fig.13b

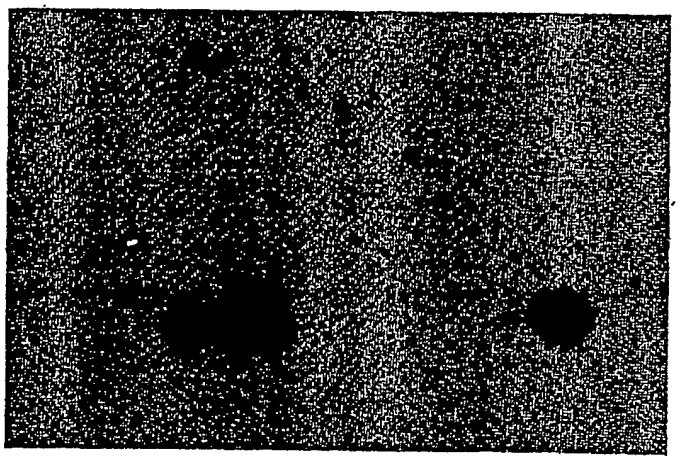


Fig.13c

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01662

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; C07H21/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCLEOSIDES NUCLEOTIDES vol. 10, 1991, pages 675 - 677 LIN, PAUL V. S. KONG THOO ET AL. 'Synthesis and stability of oligonucleotides containing purine base analogues' cited in the application see the whole document ---	1-7
A	NUCLEIC ACIDS RES., 17(24), 10373-83 1989, ARLINGTON, VIRGINIA US LIN, P. KONG THOO ET AL. 'Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogs' see the whole document --- -/--	1-7

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 DECEMBER 1992	13. 01. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MOLINA GALAN E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	FEBS LETTERS. vol. 264, no. 2, May 1990, AMSTERDAM NL pages 193 - 197 G. C. HUANG ET AL. 'Molecular cloning of a human thyrotropin receptor cDNA fragment' see abstract	4
A	EP,A,0 176 396 (INSTITUT PASTEUR) 2 April 1986	
A	WO,A,9 003 443 (CETUS CORP.) 5 April 1990	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201662 SA 64375

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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EP-A-0176396	02-04-86	FR-A- 2569407	28-02-86
		JP-A- 61069788	10-04-86
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